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Network analysis of microRNAs, transcription factors, and target genes involved in axon regeneration*#

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Abstract: Axon regeneration is crucial for recovery from neurological diseases. Numerous studies have identified several genes, microRNAs (miRNAs), and transcription factors (TFs) that influence axon regeneration. However, the regulatory networks involved have not been fully elucidated. In the present study, we analyzed a regulatory network of 51 miRNAs, 27 TFs, and 59 target genes, which is involved in axon regeneration. We identified 359 pairs of feed-forward loops (FFLs), seven important genes (*Nap1l1*, *Arhgef12*, *Sema6d*, *Akt3*, *Trim2*, *Rab11fip2*, and *Rps6ka3*), six important miRNAs (hsa-miR-204-5p, hsa-miR-124-3p, hsa-miR-26a-5p, hsa-miR-16-5p, hsa-miR-17-5p, and hsa-miR-15b-5p), and eight important TFs (Smada2, Fli1, Wt1, Sp6, Sp3, Smad4, Smad5, and Creb1), which appear to play an important role in axon regeneration. Functional enrichment analysis revealed that axon-associated genes are involved mainly in the regulation of cellular component organization, axonogenesis, and cell morphogenesis during neuronal differentiation. However, these findings need to be validated by further studies.

Key words: Transcription factors; miRNAs; Target genes; Axon; Network analysis
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1 Introduction

Axons are slender neuronal projections that transmit information from the cell body of a neuron around the body. Axons are also known as nerve fibers. Guidepost cells, typically other (sometimes immature) neurons, aid in the process of axon growth (Kunik, 2011).

Axon damage can result in the prevention of signal transmission, thereby contributing to the pathology of many neurological diseases (Debanne et al., 2011). Previous studies have shown that an injured adult mammalian central nervous system, rich in inhibitory proteins and glycoproteins, has no capacity to initiate axonal regeneration spontaneously (Geoffroy and Zheng, 2014). Therefore, promoting axon regeneration after injury is crucial. However, researchers have discovered that as long as the cell body of a neuron is not damaged, damaged axons can regenerate and recover function with the help of guidepost cells (Kunik, 2011). The process of axon regrowth after injury may be fundamentally similar to the process of axon growth in embryonic stages. In the past decade, many studies have shown that axon regeneration can be induced by the knockout of negative

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regulators or overexpression of positive factors in neurons (Ferguson and Son, 2011; Geoffroy and Zheng, 2014; Baldwin and Giger, 2015; McKerracher and Rosen, 2015; He and Jin, 2016). For example, knockout of phosphatase and the tensin homolog (Pten) resulted in the promotion of axon regeneration in adult corticospinal neurons (Huang et al., 2017). Overexpression of the inhibitor of DNA binding 2 (Id2) promoted axonal growth after injury (Yu et al., 2011).

Transcription factors (TFs) bind to target DNA sequences, either alone or as part of a complex, to increase or decrease gene transcription (Lee and Young, 2000). TFs play important roles in developmental processes (Lobe, 1992), signaling cascades (Osborne et al., 2001), cell cycle control (Evan et al., 1994), disease pathogenesis (Boch and Bonas, 2010), and responses to environmental stimuli (Pullamsetti et al., 2016). In recent years, studies have shown that TFs can stimulate axon regeneration via different molecular mechanisms (Venkatesh and Blackmore, 2017). MicroRNAs (miRNAs) are small, non-coding RNA molecules that degrade target mRNAs or inhibit their translation by binding to the 3' untranslated region (3' UTR) (Tan et al., 2009). miRNAs can regulate axon regeneration by controlling target gene expression (Jiang et al., 2015). Therefore, understanding how TFs and miRNAs regulate the expression of axon-associated genes is critical for the development of therapies that promote axon regeneration.

The expression of miRNAs can be mediated by TFs. TF and miRNA co-regulatory networks contain multiple feed-forward loops (FFLs) and feedback loops (Lin et al., 2015). Interactions between these loops control gene expression to ensure the most suitable response to external stimuli (Wang et al., 2016). FFLs include TFs that directly and indirectly regulate miRNA-target genes. In other words, miRNAs and TFs can co-regulate the same target genes. In this study, we investigated axon-associated TFs, miRNAs, and target genes using bioinformatics to construct a TF-miRNA co-regulatory network. In addition, we identified that FFLs were involved in axon regeneration. By analyzing these networks, we will better understand the cooperative TF-miRNA regulatory mechanisms and the role of FFLs in gene regulation, and be able to identify and describe regulatory mechanisms that could be used for promoting axon regeneration after being damaged.

2 Methods

2.1 Identification of differentially expressed genes and hierarchical cluster analysis

GSE84975 microarray data were downloaded from the Gene Expression Omnibus database (http://www.ncbi.nlm.nih.gov/geo). The GSE84975 dataset submitted on July 29, 2016 (Bigler et al., 2016) included three neuron samples (GSM2254874, GSM2254875, GSM2254876) derived from human embryonic stem cells (hESC-Neuron samples) and three axon samples (GSM2254871, GSM2254872, GSM2254873) derived from hESC-Neurons (Axon hESC-Neuron samples). The dataset was based on GPL16686 (Affymetrix Human Gene 2.0 ST Array) platform information. The data were background-corrected and standardized using Expression Console™ software (Affymetrix) (Irizarry et al., 2003).

Genes that were differentially expressed between hESC-Neuron samples and Axon hESC-Neuron samples were identified by one-way between-subjects analysis of variance using Transcriptome Analysis Console 3.1 software (Affymetrix) (Torres et al., 2015). Genes with thresholds of $\log_2$ fold-changes >2, $P<0.05$, and false discovery rates (FDRs) <0.05 were selected as differentially expressed genes (DEGs) (Table S1). Non-annotated probes were filtered out manually. Hierarchical cluster analysis of DEGs was carried out using Transcriptome Analysis Console 3.1 software (Affymetrix).

2.2 Identification of axon-related DEGs

We searched for axon-related genes in PubMed using "axon" as a search term. Genes related to axon growth, axon regeneration, and axon development were selected. Next, we identified those genes that overlapped between DEGs and axon-related genes. Finally, overlapping genes and genes in the same family as the overlapping genes were defined as Genes Cluster 1 **(**Table S2).

2.3 Axon-associated miRNAs and predictions of miRNA targets

We identified axon-related miRNAs by searching PubMed using "axon AND miRNA" as search terms. We selected miRNAs related to axon growth, axon regeneration, and axon development. We identified 51 mature miRNAs related to human axons (Table S3).

miRNA-target genes were predicted using the miRNA-target gene prediction databases miRanda and TargetScan. MiRanda software was developed to predict miRNA-target genes by bioinformatics methods. MiRanda screens the 3' UTR of mRNAs based on sequence matching, the thermal stability of the miRNA and mRNA double strand, and conservation of the target site. TargetScan was used to predict mammalian miRNA-target genes. It predicts conserved miRNA binding sites between different species based on a combination of thermodynamic models and RNA sequence analysis. The target genes identified by TargetScan and miRanda were analyzed and a total of 1852 targets were identified by both databases. These target genes were defined as Genes Cluster 2 (Table S4).

2.4 Prediction of axon-associated TFs

To select TFs that regulated axon-related DEGs, we downloaded human TFs from the transcriptional regulatory element database (TRED) (http://rulai.cshl.edu/TRED/TFlist.htm). Then, we screened for genes that overlapped between axon-related DEGs and human TFs. These were defined as TFs Cluster 1.

Genes Cluster 3 included 59 genes that overlapped between Genes Cluster 1 and Genes Cluster 2. We predicted the interactions between the TFs in TFs Cluster 1 and 51 axon-related miRNAs or 59 axon-related genes. We searched for gene or miRNA sequences near the transcriptional start site area, and then used the TRANSFACT database (Biobase) to predict TF binding sites based on the Match™ algorithm. The search algorithm uses two score values, the matrix similarity score (MSS) and the core similarity score (CSS), to evaluate the result. In this study, 1 was the cutoff value for the MSS and CSS.

2.5 FFLs and miRNA-TF-target gene networks

After identifying the regulatory relationships between miRNAs, TFs, and target genes, we used this information to construct FFLs and a miRNA-TF-target gene co-expression network. The network was constructed using Cytoscape v3.4.0 software (Shannon et al., 2003).

In this network, we selected some genes, miRNAs, and TFs that showed a high node degree and closeness centrality (Su et al., 2017b). Node degree is a measure of the number of interactions with other proteins. Closeness centrality is a measure of key node centrality in the network. The higher the degree and closeness centrality values, the more important is the protein in the network. The nodes with degree values above the average network degree value and with thresholds of closeness centrality of >0.5 were identified.

2.6 Functional enrichment analysis

To explore the functions and pathways of axon-associated target genes, we performed gene ontology (GO) enrichment analysis using the BiNGO tool of Cytoscape v3.4.0 software (Shannon et al., 2003). $P<0.01$ was set as the cutoff value.

3 Results

3.1 Screening for DEGs

A total of 4025 genes were identified as being differentially expressed between whole hESC-Neuron and Axon hESC-Neuron samples. Of these regulated genes, 1227 were up-regulated and 2798 down-regulated in Axon hESC-Neuron compared with whole hESC-Neuron samples (Table S1). Hierarchical cluster analysis showed that the three Axon hESC-Neuron samples were distributed within the Axon sample cluster and the three hESC-Neuron samples within the hESC-Neuron sample cluster (Fig. 1). This demonstrated that the data could be used directly for further analysis.

3.2 Interaction of axon-related genes and miRNAs

To explore the co-regulatory network of miRNAs and genes in axon regeneration, we selected 59 genes (Genes Cluster 3) that overlapped between Genes Cluster 1 (axon-associated genes) and Genes Cluster 2 (miRNA-target genes) (Table 1). Interactions between the 59 axon-related genes and miRNAs are shown in Table 2. Notably, one gene may be regulated by multiple miRNAs, and each miRNA can target more than one gene. For example, hsa-miR-16-5p, hsa-miR-15b-5p, and hsa-miR-137 all target cell division cycle 42 (*Cdc42*), and hsa-miR-15b-5p also targets serine/threonine kinase 3 (*Akt3*), *Cdc42*, nuclear factor of activated T-cells 3 (*Nfatc3*), nicotinamide nucleotide adenylyltransferase 2 (*Nmnat2*), paired box 2 (*Pax2*), RAB11 family interacting protein 2 (*Rab11fip2*), ribosomal protein S6 kinase A3 (*Rps6ka3*), and semaphorin 6D (*Sema6d*).

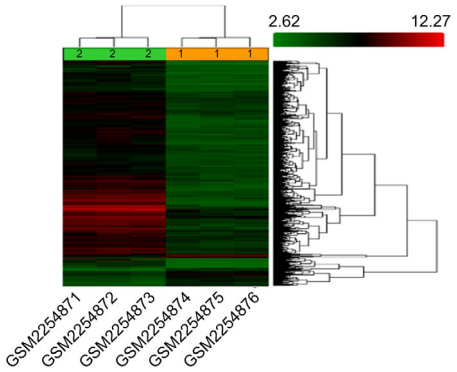

Fig. 1 Hierarchical cluster analysis of differentially expressed genes (DEGs)

The horizontal axis shows sample names. GSM2254871, GSM2254872, and GSM2254873 are Axon hESC-Neuron samples. GSM2254874, GSM2254875, and GSM2254876 are hESC-Neuron samples. The right vertical axis shows clusters of DEGs. Up-regulated genes are shown in red and down-regulated genes in green (Note: for interpretation of the references to color in this figure legend, the reader is referred to the web version of this article)

3.3 Prediction of axon-related TFs and their interactions with axon-related genes and miRNAs

TFs are proteins that can regulate miRNAs and genes. We downloaded TFs from the TRED database and identified 27 TFs in the DEG cluster; 17 were up-regulated and 10 were down-regulated (Table 3).

We predicted interactions between TFs and axon-related genes and miRNAs. We identified 18 overlapping TF interactions with 59 axon-related genes and 18 overlapping TF interactions with 51 axon-related miRNAs. The interactions between axon-related genes and TFs are shown in Table S5. One TF can regulate several genes, and one gene may be regulated by multiple TFs. The interactions between axon-related miRNAs and TFs are presented in Table S6. One miRNA can target several TFs, and TFs may in turn regulate miRNAs. Also, one TF may be regulated by multiple miRNAs.

Table 1 Axon-related and miRNA-target genes

Gene symbol	Chromosome	Fold change	*P*-value	FDR	Gene symbol	Chromosome	Fold change	*P*-value	FDR
Gsk3b	chr1	−31.75	0.000498	0.013168	*Arfgef1*	chr15	−3.84	0.001885	0.022476
Trim44	chr16	−14.62	0.001713	0.021677	*Ppp1r9a*	chr16	−3.84	0.000033	0.004571
Akt3	chr1	−12.17	0.000105	0.006809	*Sema6a*	chr1	−3.75	0.001977	0.022756
Cdc42bpa	chr19	−12.09	0.000389	0.011808	*Cdc7*	chr9	−3.58	0.010118	0.049437
Wdr33	chr9	−10.42	0.002029	0.023013	*Rpp14*	chr19	−3.55	0.009971	0.049070
Rprd1a	chrX	−10.09	0.000351	0.011204	*Ppt1*	chr10	−3.28	0.000129	0.007393
Cdc42	chr12	−8.98	0.000686	0.015027	*Rab11fip2*	chr14	−3.22	0.005658	0.036905
Nap1l1	chr9	−8.91	0.000061	0.005459	*Ccdc25*	chr1	−3.19	0.001153	0.018233
Wdr47	chr1	−8.52	0.000212	0.009014	*Ndel1*	chr2	−3.10	0.007999	0.043986
Klf12	chr1	−8.37	0.000200	0.008827	*Rps6kb1*	chr5	−3.03	0.006484	0.039601
Rap1a	chr20	−8.01	0.002805	0.026899	*Sema6d*	chr2	−2.95	0.008081	0.044224
Trim33	chr16	−7.01	0.000052	0.005198	*Cdc23*	chr1	−2.83	0.008324	0.044824
Aktip	chr17	−7.00	0.001652	0.021337	*Cdc42se1*	chr4	−2.77	0.001443	0.020183
Ppp2cb	chr4	−6.16	0.000081	0.006185	*Ppp3r1*	chr3	−2.77	0.009574	0.048062
Robo1	chr15	−5.43	0.000086	0.006299	*Tp53inp1*	chr16	−2.46	0.000496	0.013132
Arhgef12	chr3	−5.39	0.000422	0.012217	*Nfatc3*	chr7	−2.38	0.005497	0.036417
Creb1	chr7	−5.26	0.001453	0.020248	*Trim2*	chr19	−2.32	0.001761	0.021893
Rps6ka3	chr20	−5.20	0.004840	0.034406	*Nmnat2*	chr8	−2.14	0.005352	0.035897
Alcam	chr2	−4.97	0.000362	0.011369	*Trim9*	chr1	−2.12	0.000355	0.011273
Sacs	chr21	−4.90	0.005379	0.035973	*Ppp1r15b*	chr9	−2.10	0.001839	0.022286
Wdr82	chr8	−4.72	0.004450	0.033020	*Nrp1*	chrX	−2.06	0.001276	0.019120
Nova1	chr19	−4.52	0.000392	0.011870	*Sox8*	chr6	2.12	0.003220	0.028611
Ppp6c	chr15	−4.51	0.000466	0.012752	*Pax2*	chr19	2.14	0.003335	0.029089
Pten	chr3	└ 4.47	0.004651	0.033739	*Creb3l1*	chr1	2.18	0.007181	0.041678
Abce1	chr1	−4.32	0.003454	0.029535	*Cspg4*	chr19	2.18	0.008308	0.044805
Wdr26	chr2	−4.29	0.000160	0.008128	*Ldlrap1*	chr2	2.19	0.008209	0.044562
Clasp2	chr20	−4.26	0.001469	0.020333	*Wnt3a*	chr2	2.25	0.004912	0.034633
Sacm1l	chr5	−4.24	0.001135	0.018153	*Rab11fip5*	chr2	2.47	0.005251	0.035664
Ppp2r5e	chr2	−4.07	0.001179	0.018369	*Ppp1r1b*	chr5	2.57	0.001708	0.021640
Ppp4r1	chr16	−3.96	0.000083	0.006236					

FDR: false discovery rate

Table 2 Genes overlapping between Genes Cluster 1 (axon-related DEGs) and Genes Cluster 2 (axon-related miRNA-target genes) and miRNA-target gene interactions

miRNA	Gene symbol	miRNA	Gene symbol	miRNA	Gene symbol
hsa-miR-29c-3p	*Abce1*	hsa-miR-15b-5p	*Nfatc3*	hsa-miR-181b-5p	*Rps6ka3*
hsa-miR-214-5p	*Akt3*	hsa-miR-15b-5p	*Nmnat2*	hsa-miR-15b-5p	*Rps6ka3*
hsa-miR-15b-5p	*Akt3*	hsa-miR-96-5p	*Nova1*	hsa-miR-214-3p	*Rps6ka3*
hsa-miR-181d-5p	*Akt3*	hsa-miR-204-5p	*Nova1*	hsa-miR-204-3p	*Rps6ka3*
hsa-miR-541-3p	*Akt3*	hsa-miR-138-5p	*Nova1*	hsa-miR-124-3p	*Rps6kb1*
hsa-miR-17-5p	*Aktip*	hsa-miR-181d-5p	*Nova1*	hsa-miR-181b-5p	*Sacm1l*
hsa-miR-18a-5p	*Alcam*	hsa-miR-221-3p	*Nova1*	hsa-miR-181d-5p	*Sacm1l*
hsa-miR-382-5p	*Arfgef1*	hsa-miR-338-3p	*Nova1*	hsa-miR-17-5p	*Sacs*
hsa-miR-26a-5p	*Arfgef12*	hsa-miR-338-5p	*Nrp1*	hsa-miR-26a-5p	*Sacs*
hsa-miR-96-5p	*Arfgef12*	hsa-miR-338-3p	*Nrp1*	hsa-miR-214-3p	*Sema6a*
hsa-miR-214-3p	*Arfgef12*	hsa-miR-15b-5p	*Pax2*	hsa-miR-124-3p	*Sema6a*
hsa-miR-34a-3p	*Arfgef12*	hsa-miR-221-3p	*Ppp1r15b*	hsa-miR-16-5p	*Sema6d*
hsa-miR-17-5p	*Ccdc25*	hsa-miR-221-5p	*Ppp1r1b*	hsa-miR-15b-5p	*Sema6d*
hsa-miR-16-5p	*Cdc23*	hsa-miR-18a-3p	*Ppp1r1b*	hsa-miR-124-3p	*Sema6d*
hsa-miR-16-5p	*Cdc42*	hsa-miR-19a-5p	*Ppp1r9a*	hsa-miR-214-3p	*Sox8*
hsa-miR-15b-5p	*Cdc42*	hsa-miR-214-3p	*Ppp2cb*	hsa-miR-17-5p	*Tp53inp1*
hsa-miR-137	*Cdc42*	hsa-miR-132-3p	*Ppp2cb*	hsa-miR-125b-5p	*Tp53inp1*
hsa-miR-96-5p	*Cdc42bpa*	hsa-miR-221-3p	*Ppp2r5e*	hsa-miR-29c-3p	*Tp53inp1*
hsa-miR-221-5p	*Cdc42bpa*	hsa-miR-17-5p	*Ppp3r1*	hsa-miR-18a-5p	*Trim2*
hsa-miR-541-5p	*Cdc42bpa*	hsa-miR-96-5p	*Ppp3r1*	hsa-miR-181b-5p	*Trim2*
hsa-miR-19a-3p	*Cdc42bpa*	hsa-miR-204-5p	*Ppp3r1*	hsa-miR-181d-5p	*Trim2*
hsa-miR-125b-5p	*Cdc42se1*	hsa-miR-221-3p	*Ppp3r1*	hsa-miR-338-3p	*Trim33*
hsa-miR-29c-3p	*Cdc42se1*	hsa-miR-342-3p	*Ppp3r1*	hsa-miR-329-3p	*Trim33*
hsa-miR-204-5p	*Cdc7*	hsa-miR-7-5p	*Ppp4r1*	hsa-miR-17-5p	*Trim44*
hsa-miR-29c-3p	*Cdc7*	hsa-miR-17-5p	*Ppp6c*	hsa-miR-34a-3p	*Trim44*
hsa-miR-96-5p	*Clasp2*	hsa-miR-125b-5p	*Ppt1*	hsa-miR-96-5p	*Trim9*
hsa-miR-17-5p	*Creb1*	hsa-miR-26a-5p	*Pten*	hsa-miR-29c-3p	*Wdr26*
hsa-miR-34a-5p	*Creb3l1*	hsa-miR-19a-3p	*Pten*	hsa-miR-214-3p	*Wdr33*
hsa-miR-29c-3p	*Cspg4*	hsa-miR-16-5p	*Rab11fip2*	hsa-miR-29c-3p	*Wdr33*
hsa-miR-199a-5p	*Gsk3b*	hsa-miR-18a-5p	*Rab11fip2*	hsa-miR-16-5p	*Wdr47*
hsa-miR-7-5p	*Klf12*	hsa-miR-181b-5p	*Rab11fip2*	hsa-miR-7-5p	*Wdr47*
hsa-miR-221-5p	*Klf12*	hsa-miR-15b-5p	*Rab11fip2*	hsa-miR-199a-3p	*Wdr47*
hsa-miR-338-5p	*Klf12*	hsa-miR-181d-5p	*Rab11fip2*	hsa-miR-17-5p	*Wdr82*
hsa-miR-431-5p	*Klf12*	hsa-miR-7-5p	*Rab11fip5*	hsa-miR-7-5p	*Wdr82*
hsa-miR-329-3p	*Klf12*	hsa-miR-19a-3p	*Rap1a*	hsa-miR-204-5p	*Wdr82*
hsa-miR-124-3p	*Ldlrap1*	hsa-miR-29c-3p	*Robo1*	hsa-miR-181d-5p	*Wdr82*
hsa-miR-124-5p	*Nap1l1*	hsa-miR-204-3p	*Rpp14*	hsa-miR-214-3p	*Wdr82*
hsa-miR-96-3p	*Ndel1*	hsa-miR-142-3p	*Rprd1A*	hsa-miR-541-3p	*Wdr82*
hsa-miR-16-5p	*Nfatc3*	hsa-miR-16-5p	*Rps6ka3*	hsa-miR-16-5p	*Wnt3a*
hsa-miR-204-5p	*Nfatc3*	hsa-miR-17-5p	*Rps6ka3*	hsa-miR-15b-5p	*Wnt3a*

Table 3 Transcription factors overlapping between DEGs and human transcription factors (TFs Cluster 1)

Gene symbol	Chromosome	Entrez ID	Fold change	*P*-value	FDR
Hoxc13	chr12	3229	2.73	0.005219	0.035533
Relb	chr19	5971	2.40	0.001108	0.018001
Wt1	chr11	7490	2.38	0.000424	0.012237
Sp6	chr17	80320	2.34	0.000336	0.011081
Hoxd12	chr2	3238	2.32	0.005568	0.036634
Fli1	chr11	2313	2.16	0.001253	0.019005
Hoxd10	chr2	3236	2.15	0.003911	0.031160
Sp7	chr12	121340	2.15	0.006933	0.040949
Pax2	chr10	5076	2.14	0.003335	0.029089
Hoxa7	chr7	3204	2.13	0.007633	0.042904
Pou5f1	chr6	5459	2.12	0.002400	0.024910
Tlx1	chr10	3195	2.11	0.008127	0.044313
Pax5	chr9	5079	2.10	0.003859	0.030993
Hoxb7	chr17	3217	2.05	0.000383	0.011731
Tfap2e	chr1	339488	2.04	0.005196	0.035462
Tfap2b	chr6	7021	2.01	0.000176	0.008461
Hoxd8	chr2	3234	2.01	0.001178	0.018369
Sp3	chr2	6670	−2.05	0.005965	0.037936
Atf4	chr22	468	−3.30	0.005055	0.035038
Smad5	chr5	4090	−3.67	0.007672	0.043027
Atf6	chr1	22926	−4.28	0.001025	0.017499
Smad4	chr18	4089	−4.50	0.000632	0.014514
Hoxa2	chr7	3199	−4.97	0.000511	0.013316
Creb1	chr2	1385	−5.26	0.001453	0.020248
Smad2	chr18	4087	−5.31	0.001404	0.019969
Atf2	chr2	1386	−5.90	0.000344	0.011143
Sp4	chr7	6671	−6.79	0.000361	0.011366

FDR: false discovery rate

3.4 miRNA-TF-target gene network of axonal regeneration

To explore miRNA and TF co-regulatory networks involved in axon regeneration, we identified 359 FFL pairs among miRNAs, TFs, and axon-related genes (Table S7). These FFLs are illustrated in Fig. 2. Next, we constructed a network that showed the regulatory relationships among miRNAs, TFs, and target genes for axonal regeneration using Cytoscape software (Fig. 3). This network showed that one gene may be regulated by several TFs and that a TF may indirectly affect the expression of other genes by several miRNAs. This network included 109 nodes and 632 edges. Eighteen (30.5%) of the 59 axon-related genes, 39 (76.5%) of the 51 axon-related miRNAs, and 14 TFs were recruited. This miRNA-TF-target gene regulatory network may uncover new regulatory mechanisms involved in axonal regeneration.

Nodes with highly connected portions may play important biological roles in a network. Finally, we identified seven genes that were down-regulated, six miRNAs, and eight TFs including two that were up-regulated and six that were down-regulated (Table 4).

3.5 Gene functional enrichment

GO enrichment analysis revealed a significant enrichment of axon-related genes in different biological processes, including the regulation of cellular component organization, axonogenesis, cell morphogenesis during neuronal differentiation, and morphogenesis of neuronal projections (Table S8). Yellow nodes in Fig. 4 indicate significant biological processes.

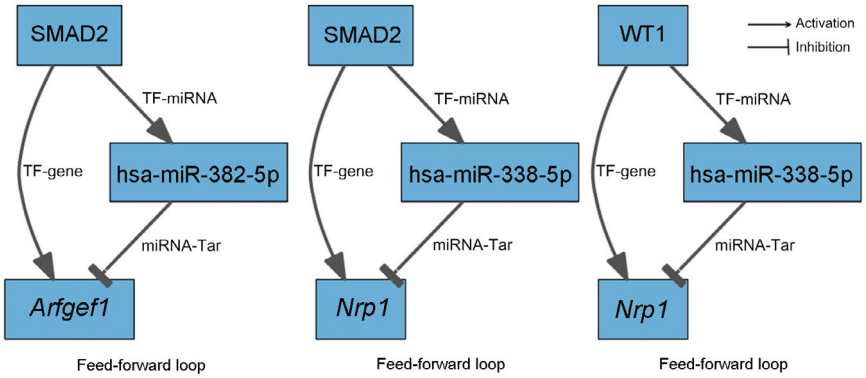

Fig. 2 Feed-forward loops among miRNAs, transcription factors, and target genes associated with axonal regeneration
Rectangles indicate miRNA, target genes or transcription factors; trigonal arrows indicate activation; blue indicates down-regulation. TF: transcription factor; Tar: target gene (Note: for interpretation of the references to color in this figure legend, the reader is referred to the web version of this article)

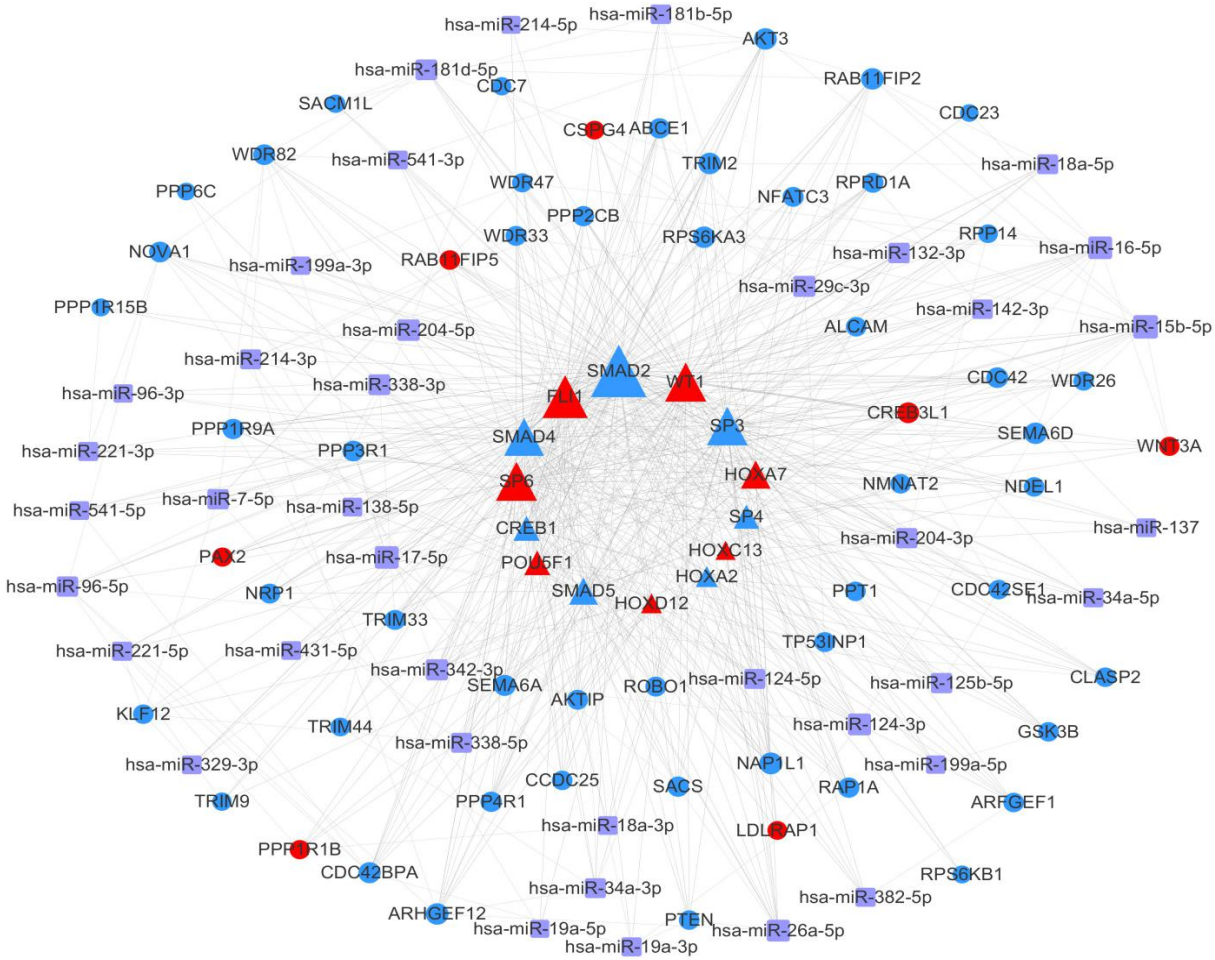

Fig. 3 Network of miRNAs, transcription factors, and target genes associated with axonal regeneration
Squares: miRNAs; circles: target genes; triangles: transcription factors. Red indicates up-regulation; blue indicates down-regulation; large nodes indicate bigger degrees (Note: for interpretation of the references to color in this figure legend, the reader is referred to the web version of this article)

Table 4 Important nodes selected based on their closeness centrality and degree in the axon regulatory network

Node	Closeness centrality	Degree	Expression	Average degree
Rps6ka3	0.52427184	12	Down	11.60
Rab11fip2	0.52173913	12	Down	
Trim2	0.51923077	12	Down	
Akt3	0.52682927	12	Down	
Sema6d	0.52173913	12	Down	
Nap1l1	0.52941176	12	Down	
Arhgef12	0.52173913	13	Down	
hsa-miR-204-5p	0.52173913	12		
hsa-miR-124-3p	0.52941176	13		
hsa-miR-26a-5p	0.52427184	13		
hsa-miR-16-5p	0.52941176	15		
hsa-miR-17-5p	0.51674641	15		
hsa-miR-15b-5p	0.53465347	17		
Creb1	0.54822335	21	Down	
Smad5	0.50000000	27	Down	
Smad4	0.60674157	54	Down	
Sp3	0.63529412	56	Down	
Sp6	0.63529412	57	Up	
Wt1	0.64670659	57	Down	
Fli1	0.72000000	67	Up	
Smad2	0.84375000	92	Down	

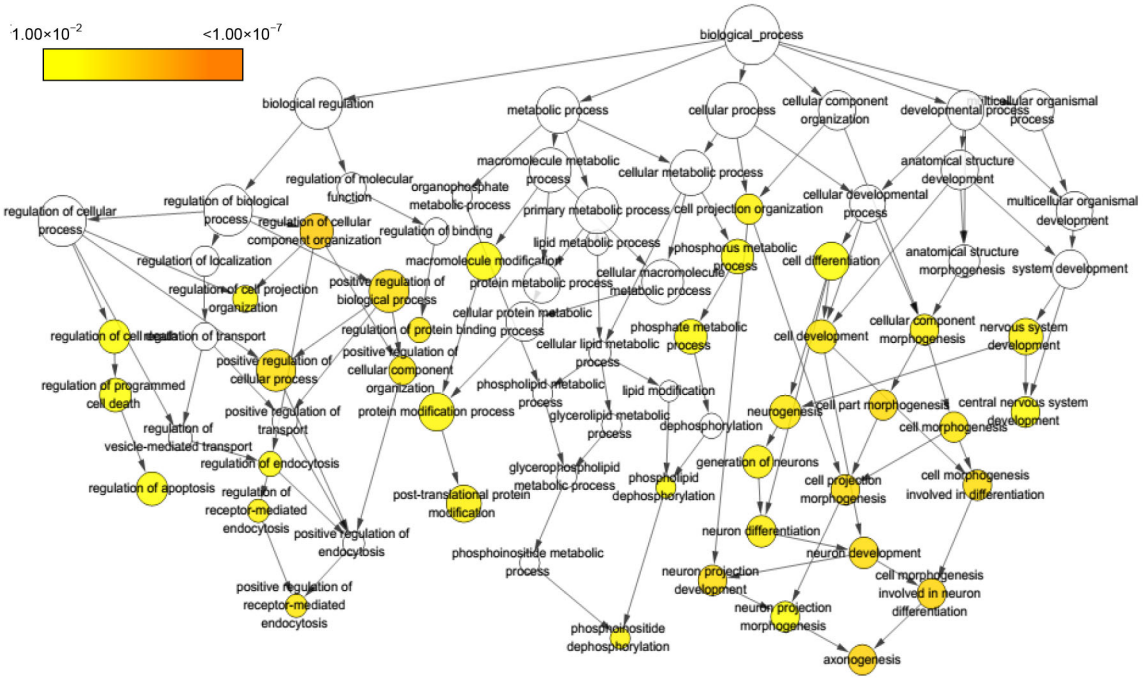

Fig. 4 Enrichment of axon-related genes in different processes displayed as a network using BiNGO software
Yellow node: $P<0.01$ (Note: for interpretation of the references to color in this figure legend, the reader is referred to the web version of this article)

4 Discussion

Many studies have found that axon regeneration after injury can be induced by altering the internal environment. Comparing the regulation relationships of axonal transcriptomes has the potential to highlight axonal regeneration mechanisms.

It is difficult to obtain nervous tissue or culture-isolated neurons, so direct identification of primary human neurons is severely restricted. Recent studies have shown that hESCs have the capacity to differentiate specific neuronal subtypes. So in this study, we used microarray data (GSE84975) related to neurons derived from hESCs and axons of hESCs differentiated into neurons from the Gene Expression Omnibus database (Bigler et al., 2016). We sought to identify the axonal genes of hESCs that had differentiated into neurons and predict the regulatory network of miRNAs, TFs, and target genes in the axon.

We investigated the regulation of genes associated with axon regeneration by TFs and miRNAs. We identified 359 FFL pairs and one regulatory network associated with axon regeneration using a bioinformatics database. In the co-regulatory network, we identified some nodes with high degree and closeness centrality, including seven genes (*Nap1l1*, *Arhgef12*, *Sema6d*, *Akt3*, *Trim2*, *Rab11fip2*, and *Rps6ka3*), six miRNAs (hsa-miR-204-5p, hsa-miR-124-3p, hsa-miR-26a-5p, hsa-miR-16-5p, hsa-miR-17-5p, and hsa-miR-15b-5p), and eight TFs (Smad2, Fli1, Wt1, Sp6, Sp3, Smad4, Smad5, and Creb1). The genes appear to be predominately down-regulated in the co-regulatory network, whereas selected TFs were up-regulated. These nodes may play important regulatory roles in axon regeneration and may further our understanding of gene regulatory mechanisms involved in axon regeneration. For example, AKT3 is the predominant AKT kinase isoform in the brain (Easton, 2005). However, Miao et al. (2016) have shown that axon regeneration is enhanced even when AKT3 is down-regulated, which suggests that high levels of AKT3 may be not required for axon regeneration. Our results were consistent with this finding. Regulatory FFL networks identified in our study suggest that AKT3 can be regulated by TFs such as POU class 5 homeobox 1 (POU5F1), Sma- and Mad-related (SMAD) family member 4 (SMAD4), SMAD family member 2 (SMAD2), Fli-1 proto-oncogene (FLI1), Wilms tumor 1 (WT1), Sp3 transcription factor (SP3), Sp6 transcription factor (SP6), and cyclic adenosine monophosphate (cAMP) responsive element binding protein 1 (CREB1), and several miRNAs including hsa-miR-15b-5p, hsa-miR-214-5p, hsa-miR-541-3p, and hsa-miR-181d-5p. The regulatory FFL networks also suggest that AKT3 acts with these TFs and miRNAs to form 17 pairs of FFLs in the related network (Table S7). For example, SP6 regulates hsa-miR-15b-5p, which targets *Akt3*.

SMAD transcription modulators regulate multiple cellular processes (Dahle and Kuehn, 2016). SMADs are divided into three classes: receptor-regulated Smads (SMAD1, SMAD2, SMAD3, SMAD5, and SMAD8/9), the common-mediator Smad (SMAD4), and inhibitory Smads (SMAD6 and SMAD7). Some members of this family have been proved to take part in the process of axon regeneration. The expression of Smad1 was induced by phosphatidylinositol 3 kinase (PI3K)-glycogen synthase kinase 3 (GSK3) signaling to prevent mammalian axon regeneration (Saijilafu et al., 2013). SMAD4 is critical for dorsal neural patterning (Chesnutt et al., 2004). Overexpression of SMAD6 blocked dorsal interneuron 1 axon outgrowth (Hazen et al., 2011). Moreover, Smad6 can inhibit the activity of the receptor-regulated Smads to regulate axon regeneration (Hazen et al., 2011). *Smad2* knockdown by RNA interference (RNAi) stimulated axonal growth in neurons (Stegmuller et al., 2008). Furthermore, SMAD2 co-operated with the ubiquitin ligase Cdh1-anaphase-promoting complex upstream of the transcriptional modulator SnoN to control axonal growth (Stegmuller et al., 2008). In the present study, we found that SMAD2 (which had the highest degree in the co-regulatory network) was down-regulated during axon regeneration, indicating a regulatory role in axonal regeneration. We showed that SMAD2 regulated 53 genes and 34 miRNAs. However, only five miRNAs (hsa-miR-125b-5p, hsa-miR-132-3p, hsa-miR-18a-5p, hsa-miR-26a-5p, and hsa-miR-541-5p) targeted SMAD2 in the network.

miR-15b is abundant in distal axons (Natera-Naranjo et al., 2010). In the present study, we showed that hsa-miR-15b-5p targets nine genes and two TFs (HOXC13 and SMAD5). Together, these two TFs targeted hsa-miR-26a-5p and nucleosome assembly protein 1 like 1 (NAP1L1).

CREB1 is a protein that binds the cAMP response element to stimulate transcription. The activation of CREB promotes the transcription of many genes associated with neuronal survival, cell differentiation and axonal growth (Freitas et al., 2013). In our previous study, we showed that CREB1 plays pivotal roles in neuronal differentiation (Su et al., 2017a). In our present study, the results showed that CREB1 plays pivotal roles in the negative regulation of axons.

Functional enrichment analysis revealed that the biological processes are regulated by genes associated with axon regeneration. These processes and the target genes are listed in Table S8. Axon regeneration-associated genes are involved mainly in the regulation of cellular component organization, axonogenesis, and cell morphogenesis during neuronal differentiation.

In conclusion, we have identified TFs, miRNAs, and target genes that are involved in axon regeneration. Furthermore, we have revealed that the regulatory relationships potentially regulate axon regeneration. However, these associations will need to be experimentally validated in future studies. Such studies will improve our understanding of the mechanisms of axon-associated miRNA-TF-target gene regulation.

Compliance with ethics guidelines

Li-ning SU, Xiao-qing SONG, Zhan-xia XUE, Chen-qing ZHENG, Hai-feng YIN, and Hui-ping WEI declare that they have no conflict of interest.

This article does not contain any studies with human or animal subjects performed by any of the authors.

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List of electronic supplementary materials

Table S1 Information of DEGs between Axon hESC-Neuron and Whole hESC-Neuron

Table S2 Overlapping genes between DEGs and genes related to axon and genes in the same family with overlapping genes (Genes Cluster 1)

Table S3 Mature miRNAs related to human axon

Table S4 Target genes of miRNAs (Genes Cluster 2)

Table S5 TFs interaction with 59 genes (TFs Cluster 2)

Table S6 TFs interaction with 51 miRNAs (TFs Cluster 3)

Table S7 Feed-forward loops in the network

Table S8 Gene ontology enrichment analysis of biological process

中文概要

题　目：神经轴突再生过程中微小 RNA（miRNA）、转录因子和靶基因的网络功能分析

创新点：本研究比较详细地阐明了与神经轴突再生相关的微小 RNA（miRNA）、转录因子和靶基因的相

互作用关系，为神经系统疾病的恢复奠定基础。

方　法：本研究从基因表达综合数据库中获得与轴突再生相关的转录因子和基因数据，利用文献报道及生物信息学数据库预测的方法筛选与轴突再生相关的基因靶向 miRNA。利用生物信息学方法分析了三者之间的网络作用关系，预测了在相互作用网络中发挥重要作用的节点。最后对目标基因的基因本体（GO）功能进行了富集。

结　论：通过分析，初步筛选了与神经轴突再生相关的 51 个 miRNA、27 个转录因子和 59 个靶标基因。进一步分析得到 359 对前馈环路，在此基础上推测了神经轴突再生过程中发挥重要作用的 7 个核心基因（*Nap1l1*、*Arhgef12*、*Sema6d*、*Akt3*、*Trim2*、*Rab11fip2* 和 *Rps6ka3*），6 个 miRNA（hsa-miR-204-5p、hsa-miR-124-3p、hsa-miR-26a-5p、hsa-miR-16-5p、hsa-miR-17-5p 和 hsa-miR-15b-5p）和 8 个转录因子（Smada2、Fli1、Wt1、Sp6、Sp3、Smad4、Smad5 和 Creb1）。

关键词：转录因子；miRNA；靶基因；神经轴突；网络分析